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VIRUS-CELL INTERACTIONS

Superinfection Exclusion between Two High-Risk Human Papillomavirus Types during a Coinfection

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ABSTRACT Superinfection exclusion is a common phenomenon whereby a single cell is unable to be infected by two types of the same pathogen. Superinfection exclusion has been described for various viruses, including vaccinia virus, measles virus, hepatitis C virus, influenza A virus, and human immunodeficiency virus. Additionally, the mechanism of exclusion has been observed at various steps of the viral life cycle, including attachment, entry, viral genomic replication, transcription, and exocytosis. Human papillomavirus (HPV) is the causative agent of cervical cancer. Recent epidemiological studies indicate that up to 50% women who are HPV positive (HPV^+) are infected with more than one HPV type. However, no mechanism of superinfection exclusion has ever been identified for HPV. Here, we show that superinfection exclusion exists during a HPV coinfection and that it occurs on the cell surface during the attachment/entry phase of the viral life cycle. Additionally, we are able to show that the minor capsid protein L2 plays a role in this exclusion. This study shows, for the first time, that superinfection exclusion occurs during HPV coinfections and describes a potential molecular mechanism through which it occurs.

IMPORTANCE Superinfection exclusion is a phenomenon whereby one cell is unable to be infected by multiple related pathogens. This phenomenon has been described for many viruses and has been shown to occur at various points in the viral life cycle. HPV is the causative agent of cervical cancer and is involved in other anogenital and oropharyngeal cancers. Recent epidemiological research has shown that up to 50% of HPV-positive individuals harbor more than one type of HPV. We investigated the interaction between two high-risk HPV types, HPV16 and HPV18, during a coinfection. We present data showing that HPV16 is able to block or exclude HPV18 on the cell surface during a coinfection. This exclusion is due in part to differences in the HPV minor capsid protein L2. This report provides, for the first time, evidence of superinfection exclusion for HPV and leads to a better understanding of the complex interactions between multiple HPV types during coinfections.

KEYWORDS coinfection, HPV16, HPV18, human papillomavirus, superinfection exclusion, virus

Coinfection of single cells with two types of the same pathogen is rare due to superinfection exclusion (SIE). SIE, also referred to as homologous interference, is the phenomenon whereby a cell infected with a specific virus type is unable to be infected by a second virus of the same or related species. It has been described as a "selfish" viral mechanism, allowing a virus to infect a cell and replicate without competition (1–3). SIE has been described for a number of viruses, including vaccinia virus (4), measles virus (5, 6), Sindbis virus (7), Semliki Forest virus (8), hepatitis C virus (9, 10), West Nile virus (11), influenza A virus (12), human immunodeficiency virus (13, 14), and rubella virus (15). The mechanisms of SIE have been identified at several distinct steps of the viral life cycle, depending on the virus, including early steps such as attachment (6, 14, 16–21) and entry into/penetration of the capsid (8, 22, 23) as well

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as subsequent steps such as RNA replication, translation of viral proteins, and budding of new virus particles (7, 24–28).

Human papillomavirus (HPV) is a DNA tumor virus that is the causative agent of cervical cancer and associated with other anogenital and oropharyngeal cancers (29–32). It is the second most common cancer in women worldwide, with the high-risk types HPV16 and HPV18 being responsible for 70% of cervical cancer cases (33–36). Recent epidemiological studies indicate that up to 50% of women infected with HPV are concurrently infected with more than one type (37–47). Evaluation of clinical samples led to the detection of multiple HPV types within single cells in cervical lesions (48, 49). It is unclear, however, whether there is a difference in HPV persistence, viral genomic transforming potential, or an increased risk of incidence or severity of cervical intraepithelial neoplasia resulting from a coinfection (37, 50–52). Mechanistically, the possible interactions or interferences resulting from multiple HPV genotypes interacting in a HPV coinfection have yet to be investigated.

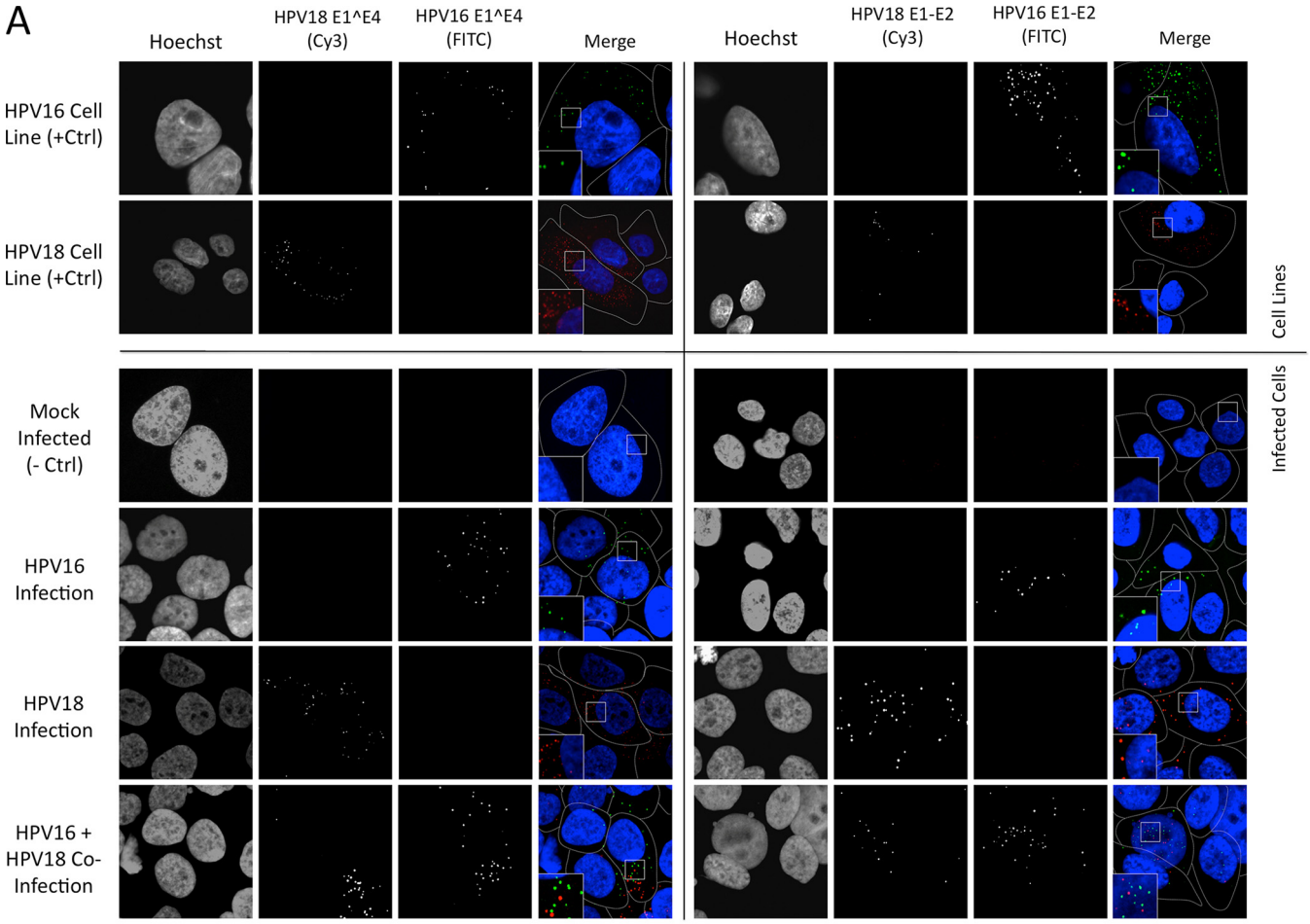
Papillomaviruses are nonenveloped and contain a circular, double-stranded DNA genome that is approximately 8 kb long. The capsid consists of two structural proteins, the major capsid protein L1 and the minor capsid protein L2. The life cycle of HPV is tightly linked to the differentiation of host cells. Microabrasions in the tissue allow the virus to gain access to basal cells (53), where the virus can bind to the extracellular matrix (ECM) and/or the cell surface via L1 (54–59). Entry has been shown to involve multiple receptors/coreceptors, including heparan sulfate (HS) (54, 57–60), $\alpha 6\beta 4$ integrin (61–65), growth factor receptors (66, 67), and annexin-A2 (67–69). Upon infection, the genome is maintained within the basal cells (70). As cells divide and differentiate, the genome is replicated, and early proteins involved in viral replication, cellular transformation, and early gene expression are expressed (71, 72). As the cells reach terminal differentiation, the late structural proteins L1 and L2 are expressed, facilitating the assembly of new virions (70).

In this study, we use native HPV16 and HPV18 virions, produced in the organotypic raft culture system, to examine the ability of single cells to be coinfected with more than one HPV type. Additionally, we investigate whether there is competition or SIE during a HPV coinfection. We observed that single cells were readily able to be coinfected with HPV16 and HPV18. However, there was a marked decrease in HPV18 infectivity in the presence of HPV16 compared to that with a single infection. Further analysis revealed that the competition/interference occurred early in the infectious process, at the level of viral attachment to the ECM/cell surface. Previous reports indicate that there is a differential dependence on heparan sulfate proteoglycan (HSPG) usage as well as the proprotein convertase furin, which cleaves L2, between HPV16 and HPV18 (73–75). To determine which capsid protein was responsible for the block of HPV18 attachment, chimeric viruses were generated by swapping the HPV16 and HPV18 L1 and L2 open reading frames. We determined that HPV16 L2 is at least partially responsible for the ability of HPV16 to block HPV18 attachment to cells. These data provide important insight into the interaction of multiple HPV types during a coinfection.

RESULTS

Individual cells can be infected with more than one HPV type simultaneously. In the xenograft system, tissue can be coinfected with more than one HPV type. However, the infections remain in regionally separate areas of tissue, and single cells do not harbor more than one HPV type (76). This is in line with previously reported data for other viruses, whereby a variety of mechanisms of SIE prevent single cells from being coinfected with more than one virus type (4–12, 14–19, 22, 24, 77, 78). To determine whether SIE is present for HPV or whether single cells could be infected with more than one HPV type simultaneously, we used RNA fluorescent *in situ* hybridization (RNA-FISH). This also allowed the analysis of transcriptional activity within infected cells. The probes utilized detected either the E1^E4 splice transcript or E1 and E2 transcripts

A



B

	Percent of Cells Infected
HPV16 Only Infections	77.9 ± 18.5
HPV18 Only Infections	76.4 ± 15.3
Co-Infections - Total Cells Infected	81.4 ± 17.1
HPV16 Only Positive Cells	17.6 ± 11.3
HPV18 Only Positive Cells	16 ± 11.5
HPV16 and HPV18 Positive Cells	47.8 ± 19.4

FIG 1 A single cell can be infected with multiple HPV types. (A) HaCaT cells were infected with HPV16 only, HPV18 only, or HPV16 and HPV18 together, and E1^E4 (left) and E1-E2 (right) mRNAs were detected via RNA-FISH. HPV16 mRNA is labeled with fluorescein isothiocyanate (FITC) and depicted in green, and HPV18 mRNA is labeled with Cy3 and depicted in red in the merged image. Nuclei are stained with Hoechst dye and depicted in blue in the merged image. Individual channels are shown in grayscale. The inset in the merged image is representative of a magnified portion of the merged image (indicated by a small white box within the image). (B) Quantitation of infected cells via RNA-FISH staining. All experiments were done two times with two different virus preparations. These results are representative of data from at least 40 images taken per experiment.

in infected cells. HaCaT cells were infected with HPV16 and/or HPV18 and then stained by FISH to detect mRNA transcripts (Fig. 1).

As positive controls, FISH was performed on HPV-positive (HPV^+) cell lines that stably maintain either the HPV16 or the HPV18 genome. As a negative control, FISH was performed on mock-infected HaCaT cells. In samples with single infections, we were able to detect cells in which either HPV16 or HPV18 was transcriptionally active with

both the E1∧E4 and E1-E2 RNA probes (Fig. 1A, fourth and fifth rows). Infection with only HPV16 resulted in 77.9% of cells being infected, and infection with only HPV18 resulted in infection of 76.4% of cells. Within coinfected samples, there was a heterogeneous population of infected cells, with 17.6% of cells being infected with HPV16 only, 16.0% of cells being infected with HPV18 only, and 47.8% of cells being coinfected with HPV16 and HPV18 (Fig. 1B). However, we did not quantitate the number of individual molecules of E1∧E4 or E1-E2. These data confirm that at least two HPV types can infect a single cell and be transcriptionally active within the same cell.

Coinfection with HPV16 and HPV18 decreases HPV18 E1∧E4 transcription. Many viruses exhibit at least one mechanism of SIE during a coinfection, preventing single cells from being infected by more than one virus type (4–20, 22, 24, 78). Epidemiological studies have determined that up to 50% of women who are infected with HPV are concurrently infected with more than one type (37–47, 79). However, whether HPV exhibits any mechanisms of SIE or whether HPV types compete during a coinfection has yet to be demonstrated. To determine whether two high-risk types had any effect on each other during a concurrent coinfection, both HaCaT cells (Fig. 2A) and primary keratinocytes (Fig. 2B) were infected with either HPV16, HPV18, or both types. The E1∧E4 splice transcript was amplified in a reverse transcription-quantitative PCR (qRT-PCR) assay as a measure of infectivity. In HaCaT cells, there was a significant decrease in HPV18 E1∧E4 transcription in the presence of HPV16, compared to a single HPV18 infection (Fig. 2A). The decreased transcription was more dramatic in primary cells, with an ~70% reduction in HPV18 E1∧E4 transcription (Fig. 2B). As controls to ensure that cellular debris in the virus preparations was not the cause of the block in HPV18 infectivity, we repeated the coinfections with OptiPrep-purified virions (Fig. 2C), and we infected the cells with a single HPV type in the presence of a primary human foreskin keratinocyte (HFK) lysate, which contains all of the cellular debris found in an organotypic raft but no virus (Fig. 2D). There was no observable decrease in HPV18 infectivity in HPV16/HPV18 coinfections with quasivirions (QVs) (Fig. 2E). Additionally, coinfections with HPV virus-like particles (VLPs) were unable to block infection by the opposite HPV type at a concentration calculated to be equivalent to that of a native virus (NV) infection at a multiplicity of infection (MOI) of 10. However, an increase in the VLP concentration led to a slight decrease in NV infectivity (Fig. 2F). These experiments verify that cellular debris in the virus preparations is not contributing to the decrease in HPV18 infectivity shown in Fig. 2A and B. Overall, these data indicate that while both types are able to simultaneously infect single cells, there is some mechanism of SIE or competition between virus types during a coinfection.

HPV18 infectivity is not recovered by the addition of surplus HPV18 virions or by preaddition of HPV18 during a coinfection. To determine whether the level of HPV18 infectivity could be recovered to the levels with a single HPV18 infection during a coinfection, we first performed coinfections in the presence of increasing amounts of HPV18 with a constant MOI of HPV16 (Fig. 3A). Infections in the presence of HPV16 (constant MOI of 10) with HPV18 at MOIs of 10, 20, and 50 were still significantly reduced compared to single HPV18 infections at these same MOIs. In contrast, once we added at least 10-fold more HPV18 virions to cells than the number of HPV16 virions, there was no longer a significant block in HPV18 infectivity (Fig. 3A, left). As noted above, the infectivity of HPV16 was unchanged in the presence of HPV18, regardless of the MOI of HPV18 added (Fig. 3A, right). Recent studies have shown that HPV16 and HPV18 have different requirements for binding to cells prior to an infection (73, 75). HPV16 is able to enter cells independently of HS (73, 75). However, HPV18 infectivity is dependent on HS. Additionally, HPV16 can enter cells independently of furin, whereas HPV18 is dependent on furin activity (74). We therefore wanted to test whether the addition of HPV18 prior to the addition of HPV16 would allow the recovery of HPV18 infectivity in the presence of HPV16. HPV18 was added to cells at 4°C for 1.5 h, allowing binding but not entry. Cells were then washed to remove any unbound particles. We then added HPV16 at 4°C and incubated the cells for an additional 1.5 h, followed by another wash to remove unbound particles. Cells with attached virus were incubated

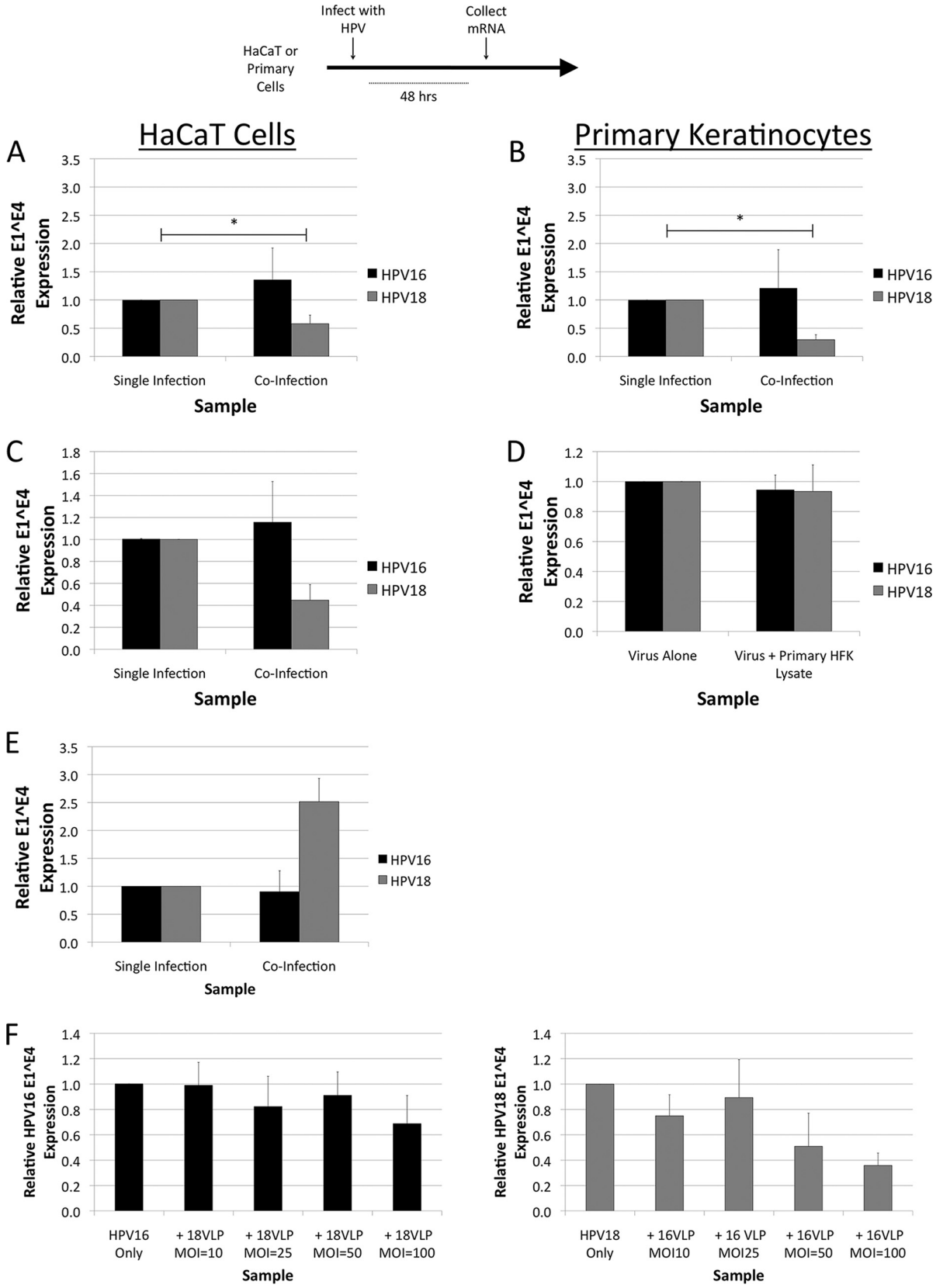


FIG 2 HPV18 infectivity is decreased in the presence of HPV16 during a coinfection. (A and B) HaCaT cells (A) or primary foreskin and cervical keratinocyte cells (B) were infected with HPV16, HPV18, or both HPV16 and HPV18. At 48 hpi, mRNA was harvested, and levels of the E1∧E4 splice transcript were determined by qRT-PCR. The levels of infection are relative, with values for single infections being set equal to 1. (C) HaCaT cells infected with OptiPrep-purified HPV16, HPV18, or both HPV16 and HPV18. At 48 hpi, mRNA was harvested, and levels of the E1∧E4

(Continued on next page)

for 48 h at 37°C to allow infection. mRNA was then harvested, and the infections were analyzed via qRT-PCR amplifying the E1∧E4 splice transcript. The level of HPV18 infectivity was unable to be recovered, even when HPV18 was added prior to the addition of HPV16 (Fig. 3B). These data suggest that, in agreement with data from previous attachment studies (73–75), HPV16 and HPV18 may initially differentially bind to the surface of cells or have differential entry requirements, leading to competition or SIE during a coinfection.

HPV16 blocks HPV18 infection at early time points in infection but not in HPV$^+$ persistent cell lines. We next sought to determine whether HPV18 infectivity would be blocked or knocked down in cells acutely infected with HPV16. In cells infected with HPV16 for 24 h followed by the addition of HPV18, HPV18 infectivity was still significantly decreased (Fig. 3C, top right). In contrast, infection of cells with HPV18 followed by infection 24 h later with HPV16 resulted in no significant decrease in HPV18 infectivity (Fig. 3C, top left), indicating that if HPV18 is able to enter cells and establish an infection, the addition of HPV16 no longer affects HPV18 infectivity. There was no difference in HPV16 infectivity regardless of whether it was added to cells pre- or post-HPV18 infection (Fig. 3C, bottom).

We then wanted to look at whether HPV18 could efficiently infect an established cell line harboring a persistent HPV16 infection. Cells that persistently maintain episomal copies of the HPV16 genome (HPV16$^+$ cells) were infected with increasing MOIs of HPV18. At 48 h postinfection (hpi), mRNA was harvested to determine the levels of HPV16 and HPV18 E1∧E4. HPV18 was able to efficiently infect HPV16$^+$ cells in a dose-dependent manner, and there was no observed block in HPV18 infectivity (Fig. 3D). Additionally, cells that persistently maintain the HPV18 genome (HPV18$^+$ cells) were infected with increasing amounts of HPV16. As expected, HPV16 was able to efficiently infect HPV18$^+$ cells in a dose-dependent manner (Fig. 3E). Infection of HPV18$^+$ cells with HPV16 did not lead to a decrease in HPV18 gene expression (Fig. 3E). Interestingly, when we compared the abilities of HPV16 and HPV18 to infect primary cells and persistently infected cells, HPV18 was approximately 23-fold more efficient at infecting persistently infected HPV16$^+$ cells than primary, HPV-negative (HPV$^-$) cells (Fig. 3G). This is possibly due to a change in the ECM composition of primary cells and established cell lines or changes in the nuclear environment. HPV16 was equally efficient at infecting primary cells and persistently infected HPV18$^+$ cells (Fig. 3F). These data support the finding that once there is an established HPV18 infection, the addition of HPV16 no longer has an effect on HPV18 infectivity.

Transcription levels of HPV16 and HPV18 are equal during a cotransfection. Mechanisms of SIE have been identified at various points in the viral life cycle, including both early and late events in infection. There are three main stages during HPV infectivity where interaction or competition between HPV16 and HPV18 could be taking place: at the level of attachment and entry at the cell surface, at the level of genome amplification, or at the level of transcription. To analyze whether the block in HPV18 infectivity during a coinfection was at the level of transcription, we transfected cells with HPV16 and/or HPV18 genomes. This allowed the equal measurement of only transcription and disregarded any events that occur prior to the delivery of the genome

FIG 2 Legend (Continued)
splice transcript were determined by qRT-PCR. The levels of infection are relative, with values for single infections being set equal to 1. (D) HaCaT cells coinfected with either HPV16 or HPV18 and the primary HFK lysate. At 48 hpi, mRNA was harvested, and the levels of the E1∧E4 splice transcript were determined by qRT-PCR. The levels of infection are relative, with values for single infections being set equal to 1. Panels A to D are representative of results from at least three individual experiments utilizing at least two different HPV16 and HPV18 preparations. (E) HaCaT cells were infected with OptiPrep-purified HPV16 and/or HPV18 QV at a MOI of 10. At 48 hpi, mRNA was harvested, and levels of the E1∧E4 splice transcript were determined by qRT-PCR. The levels of infection are relative, with values for single infections being set equal to 1. (F) HaCaT cells were coinfected with either HPV16 NV (MOI = 10) and HPV18 VLPs (equivalent to MOIs of 10, 25, 50, and 100) (left) or HPV18 NV (MOI = 10) and HPV16 VLPs (equivalent to MOIs of 10, 25, 50, and 100) (right). Both NVs and VLPs were OptiPrep purified. At 48 hpi, mRNA was harvested, and the levels of the E1∧E4 splice transcript were determined by qRT-PCR. The levels of infection are relative, with values for single infections being set equal to 1. Panels E and F are representative of data from at least 2 different experiments with at least 2 batches of NV, QV, and VLPs. Bars represent standard deviations. An asterisk denotes significance by Student's *t* test. Statistical significance was defined as a *P* value of $\leq$0.05.

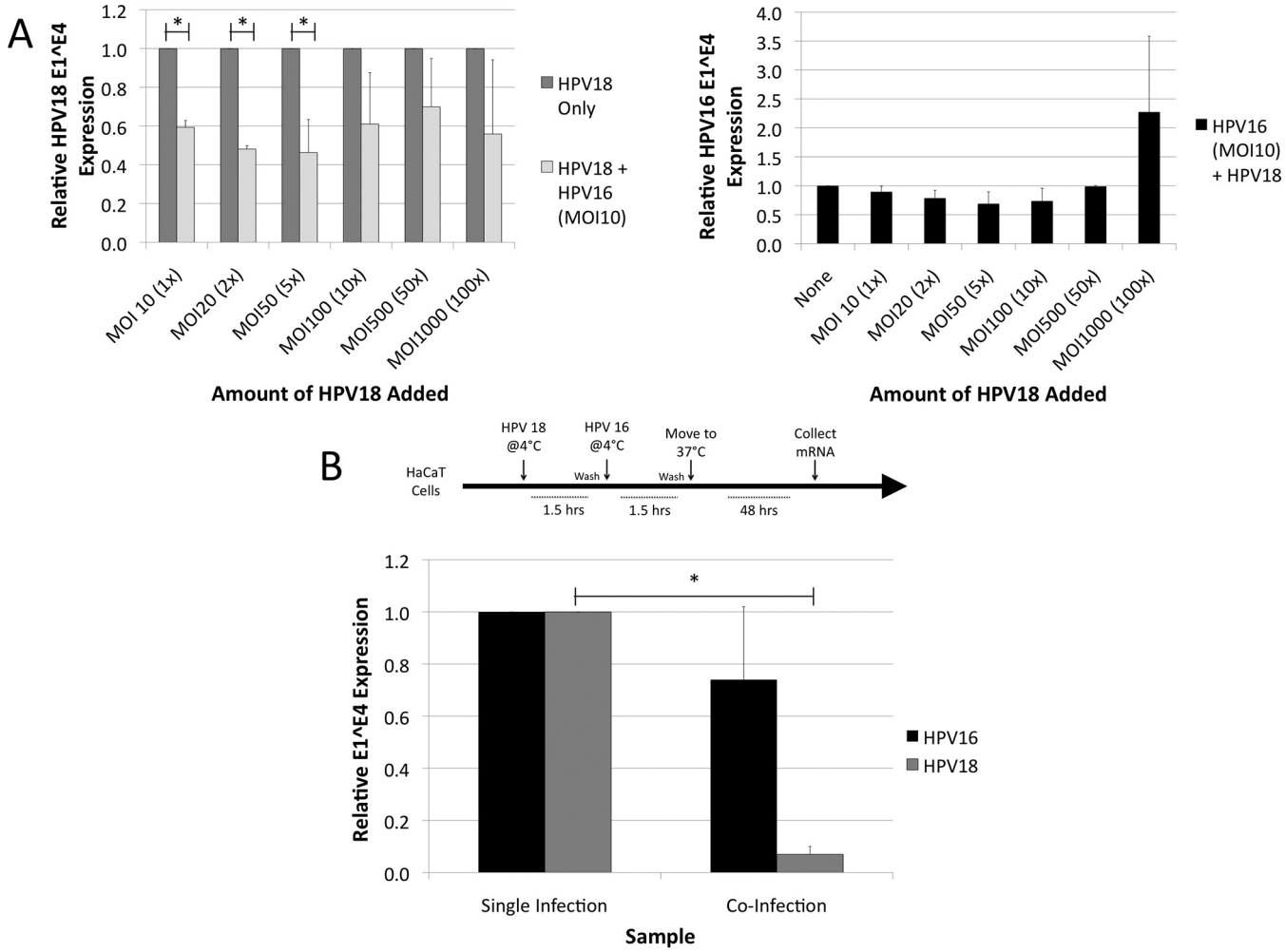


FIG 3 HPV18 infectivity is recovered by infection with additional virions but not with prebinding. (A) HaCaT cells were infected with HPV16 only (MOI = 10), HPV18 only (MOI = 10 to 1,000), or HPV16 (MOI = 10) and HPV18 (MOI = 10 to 1,000) together, followed by harvesting of mRNA and measurement of infectivity via qRT-PCR amplifying the E1∧E4 splice transcript. Analysis was performed on both HPV18 samples (left) and HPV16 samples (right). Values for individual HPV18 infections at each MOI were set equal to 1, and the relative levels of the HPV18 transcript detected in coinfections are shown. The levels of HPV16 during coinfections are all measured relative to the level of E1∧E4 detected during a single HPV16 infection. (B) HPV18 (MOI = 10) was bound to HaCaT cells at 4°C, followed 1.5 h later by the addition of HPV16 (MOI = 10) at 4°C. RNA was harvested, and infectivity was measured via qRT-PCR amplifying the E1∧E4 splice transcript. (C) HaCaT cells were seeded as described above. The cells were then infected at time zero with either HPV16 or HPV18. At 24 h, uninfected cells were infected with either HPV16 or HPV18 and a subset of the infected cells was coinfected with the other HPV type. RNA was harvested, and the level of infection was measured via qRT-PCR as described above. (D and E) Cells that stably maintain the HPV16 (HPV16 WT:3)or HPV18 (HPV18c) genome were seeded and then infected with increasing MOIs of HPV18 and HPV16, respectively. RNA was then harvested, and the levels of E1∧E4 present for both HPV types were assessed via qRT-PCR as described above. (F and G) Primary cells and stable cell lines harboring either HPV18 or HPV16 were infected with HPV16 or HPV18, respectively, at a MOI of 10. RNA was harvested, and infections were analyzed by determining E1∧E4 splice transcript levels via qRT-PCR. The level of infection in primary cells was set equal to 1, and the level of infection in stable cell lines persistently maintaining a HPV genome was measured relative to primary cell infections. All data are representative of results from at least three individual experiments utilizing at least two different HPV16 and HPV18 preparations. Bars represent standard deviations. An asterisk denotes significance by Student's *t* test. Statistical significance was defined as a *P* value of ≤0.05.

to the nucleus. Transfection of HPV16 DNA only and HPV18 DNA only resulted in nearly equal threshold cycle (C_T) values when analyzed by quantitative PCR (qPCR) against a standard curve of known DNA concentrations, indicating an equal level of transfection efficiency. At 24 h, 48 h, and 72 h posttransfection, mRNA was harvested from transfected cells, and qRT-PCR was performed, amplifying the E1∧E4 splice transcript as a measure of transcription (Fig. 4). At both 24 and 48 h after cotransfection of HPV16 and HPV18 DNAs, significant decreases in both HPV16 and HPV18 transcript levels were observed.

However, by 72 h after cotransfection, there was no longer a significant difference in the transcript levels of either HPV16 or HPV18 E1∧E4 (Fig. 4). At early time points after cotransfection, it is likely that both genomes efficiently enter the nucleus of cells and compete for transcription machinery, leading to decreased levels of transcripts for

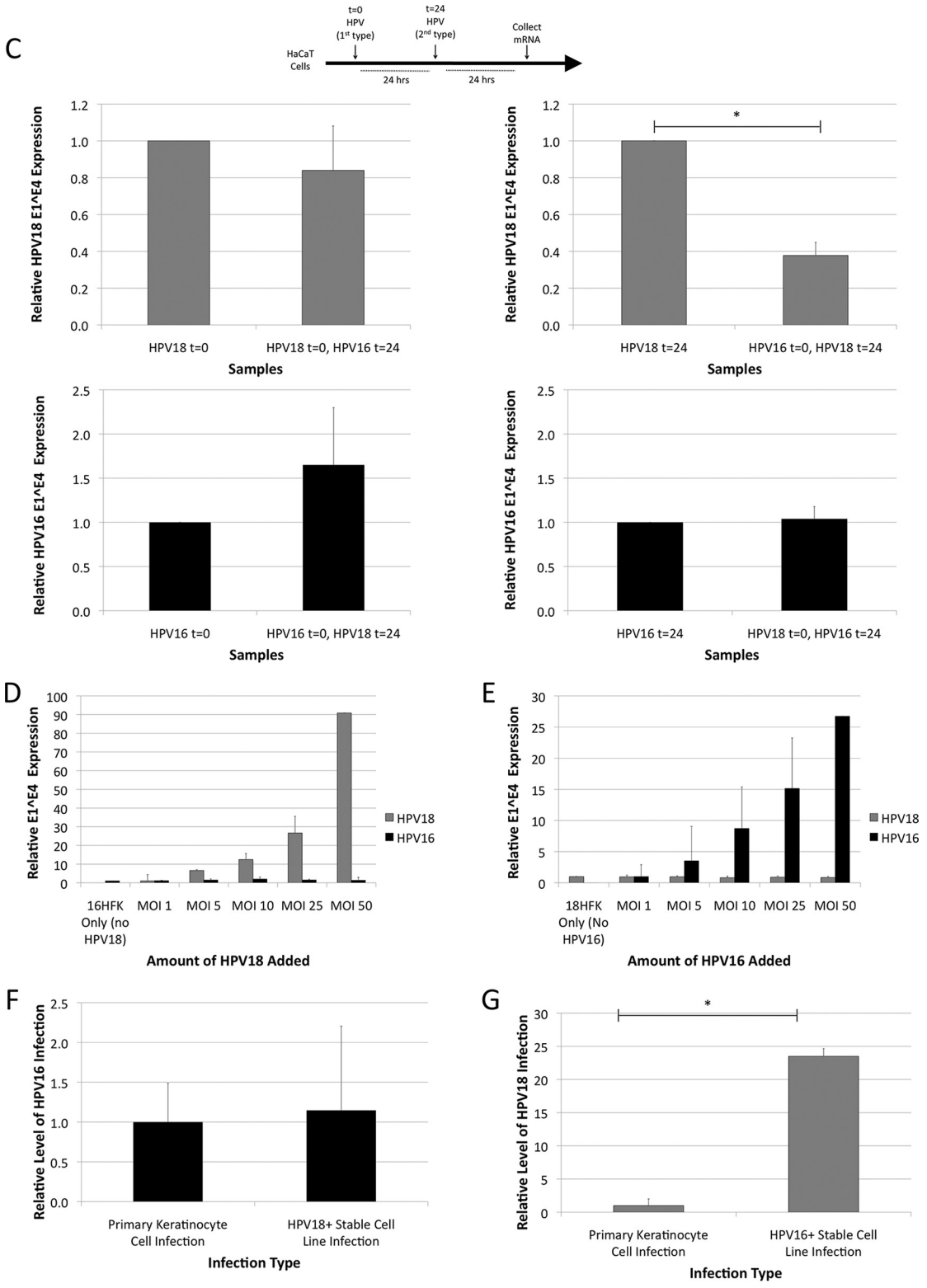


FIG 3 (Continued)

both HPV types. After 72 h, both HPV types have equally hijacked the cellular transcription machinery and are able to transcribe mRNA equally efficiently. This suggests that when both genomes reach the nucleus at the same time, there is equal competition at the level of transcription between HPV16 and HPV18.

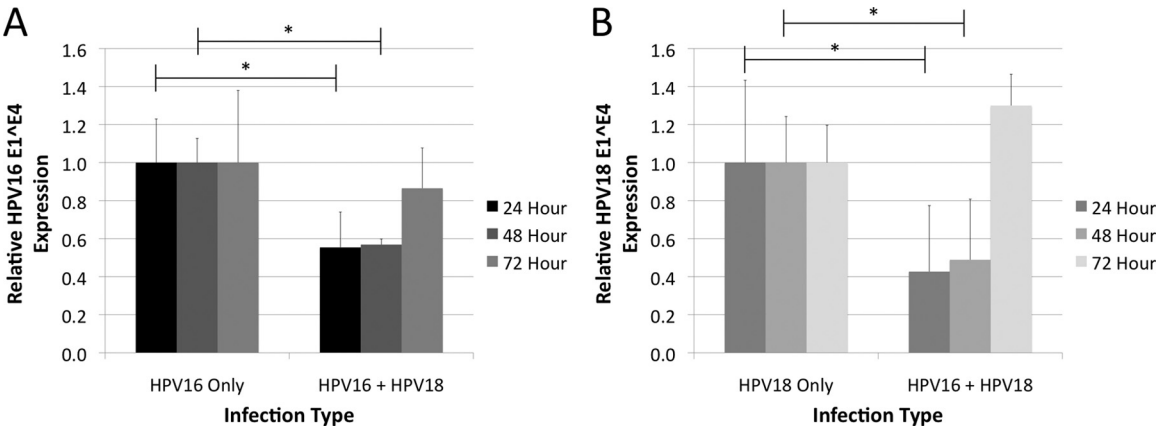


FIG 4 HPV16 does not block HPV18 during transcription. HaCaT cells were transfected with HPV16 only, HPV18 only, or both HPV16 and HPV18 genomic DNAs. At the indicated time points posttransfection, mRNA was harvested, and qRT-PCR was utilized to detect the amount of the E1^E4 splice transcript as a measure of active transcription. The level of transcription measured during cotransfections at each time point is shown relative to the level of transcription during transfection with a single HPV type. Transfections were all done in triplicate. Bars represent standard deviations. An asterisk denotes significance by Student's *t* test. Statistical significance was defined as a *P* value of $\leq$0.05.

HPV16 blocks attachment of HPV18 to cells. We next wanted to determine whether HPV16 was blocking the attachment of HPV18 to the cell surface or ECM, prior to entry. HPV16 and/or HPV18 was added to HaCaT cells or primary human keratinocytes at 4°C for 1.5 h, to allow attachment but not internalization. Cells were then washed to remove unbound virions, and the number of genomes bound per cell was quantified by qPCR. In both HaCaT cells (Fig. 5A) and primary keratinocyte cells (Fig. 5B), there was a significant reduction in the quantity of HPV18 particles bound to cells in the presence of HPV16 compared to the quantity bound to cells with only HPV18. To determine the efficiency of the simultaneous binding of HPV16 and HPV18 to the same cell, virus was bound to cells, fixed, and then stained by utilizing HPV type-specific antibodies against L1 (Fig. 5C). To ensure that no cross-reactivity was present with the antibodies, cells that had only HPV16 or only HPV18 added were stained with both the HPV16 and HPV18 L1 antibodies. No cross-reactivity was identified. Visualization of the quantification of attachment via immunofluorescence (Fig. 5D) confirms what we have reported via qPCR. In cells where HPV16 and HPV18 are bound to the cell surface, there is an ~90% decrease in the number of HPV18 virions bound compared to cells that have only HPV18. Taken together, these data indicate that HPV16 and HPV18 can bind to the same cell and that HPV16 interferes with HPV18 attachment to cells, on either the cell surface or the ECM, during a simultaneous coinfection.

HPV16 and HPV18 have different binding localization patterns and internalization times. Previously, all HPV types were shown to bind to HSPGs for attachment to either the cell surface or the ECM, allowing for conformational changes in the viral capsid, prior to the virus binding to its still unidentified entry receptor (56, 80–83). Those studies were all reliant on the use of recombinant HPV particles generated in an overexpression system whereby capsid proteins self-assemble into virus-like particles. More recently, however, in studies utilizing both recombinant and native virions, data have shown that not all HPV types have the same attachment requirements (59, 73, 75). Data indicate that both HPV16 NV and HPV16 pseudovirus (PsV) can infect cells in the absence of HSPGs (73, 75). In contrast, HPV18 NV is dependent on the presence of HSPGs for infection (73, 75). We therefore wanted to investigate whether HPV16 and HPV18 differentially bind to cells. To look at attachment to the cell surface only, an attachment assay was done with HaCaT cells in suspension, thereby removing the ECM and allowing attachment to the cell surface only (Fig. 6A). Additionally, cells were seeded and then treated with 10 mM EDTA to remove the cells and leave their ECM behind, allowing virus attachment to the ECM only (Fig. 6A and B) (55). To measure the

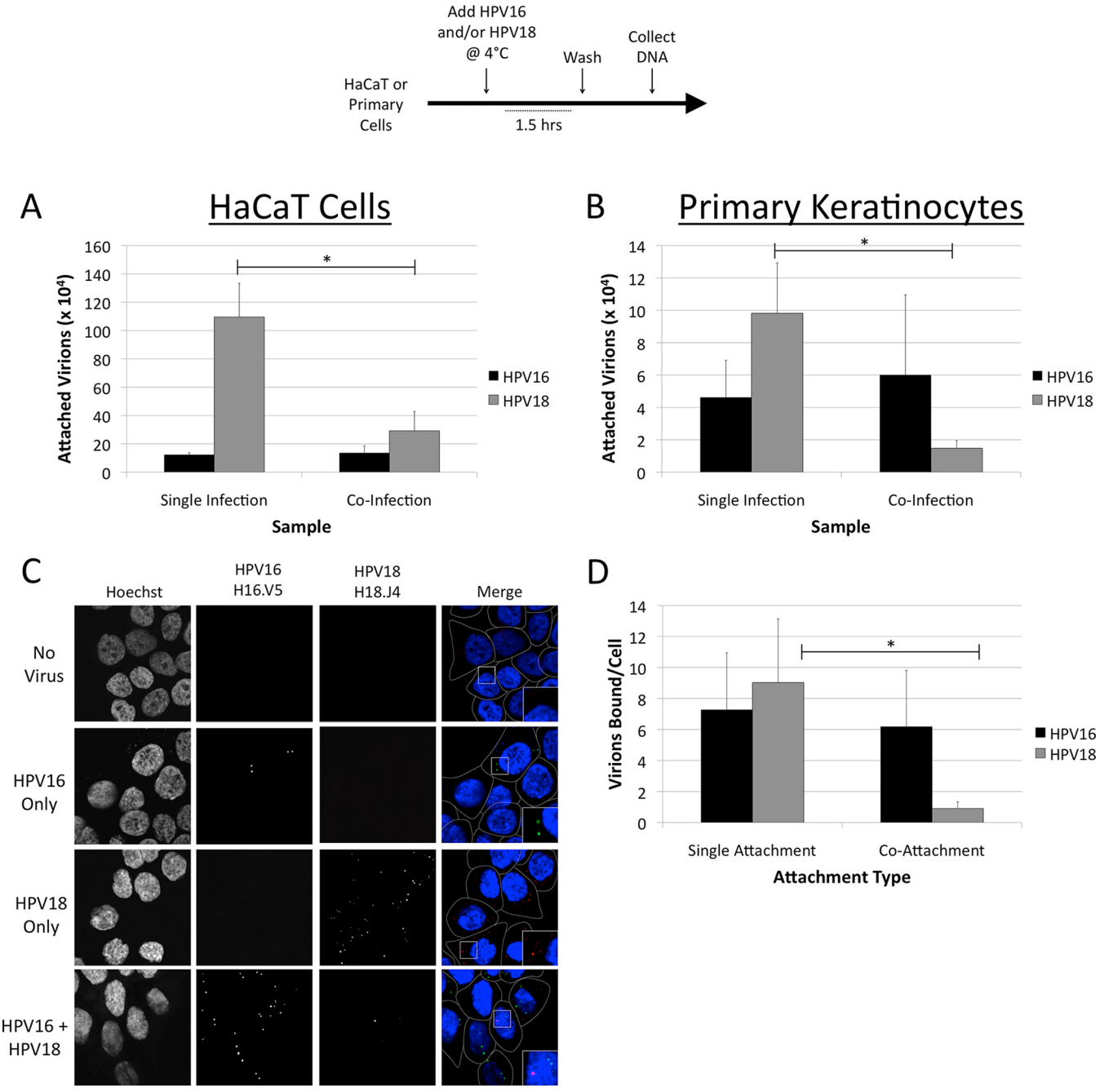
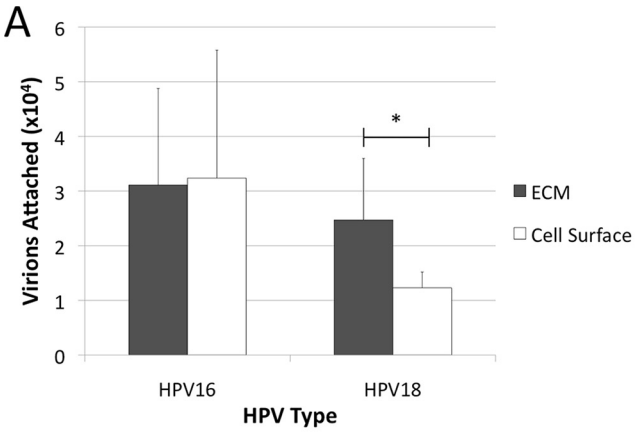
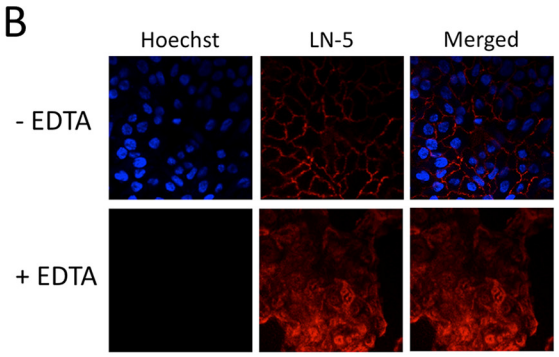
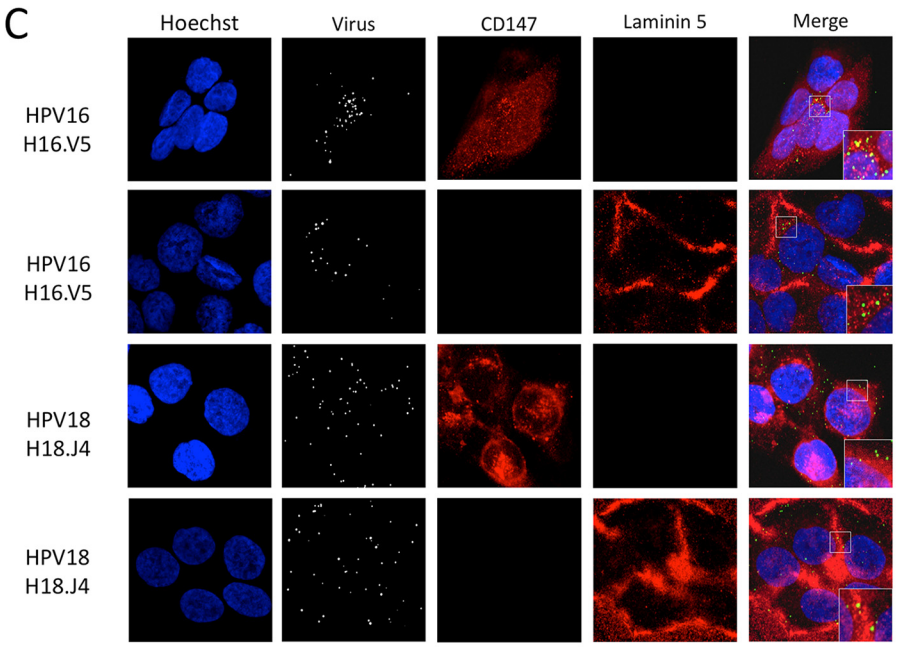


FIG 5 HPV16 blocks attachment of HPV18. (A and B) HaCaT cells (A) or primary keratinocytes (B) were incubated with HPV16, HPV18, or both types for 2 h at 4°C. Cells were then washed to remove any unbound particles, and total DNA was harvested. The number of virus particles bound to cells was quantified via qPCR against a standard curve of known concentrations of HPV genomes. (C) HaCaT cells were incubated with HPV16, HPV18, or both types for 2 h at 4°C. Cells were then washed, fixed, and stained for HPV16 and/or HPV18 L1 to detect virions bound to the cell surface. The inset in the merged image is representative of a magnified portion of the merged image (indicated by a small white box within the image). (D) Quantitation of HPV16 and HPV18 attachment to cells. Panels A and B are representative of data from at least three individual experiments, and panels C and D are representative of data from two individual experiments, all utilizing at least two different HPV16 and HPV18 preparations. Bars represent standard deviations. An asterisk denotes significance by Student's *t* test. Statistical significance was defined as a *P* value of ≤ 0.05.

level of binding to either the cell surface or ECM only, DNA was harvested after attachment, and the number of genomes bound was quantified via qPCR. HPV16 was equally efficient at binding to the cell surface and the ECM. In contrast, HPV18 bound more efficiently to the ECM of cells than to the cell surface directly, with approximately two times more particles binding to the ECM than to the cell surface (Fig. 6A). To further







D

	Percent Attachment
HPV16 Attachment	
Cell Surface	82.1 ± 11.1
ECM	14.7 ± 8.0
HPV18 Attachment	
Cell Surface	29.6 ± 12.5
ECM	73.7 ± 7.1

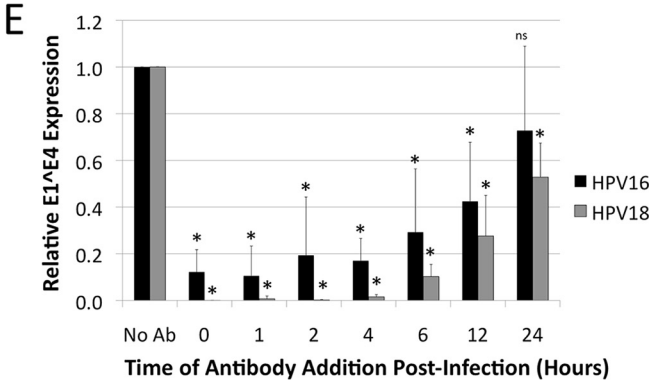


FIG 6 HPV16 and HPV18 have differential binding abilities and entry times. (A) Attachment to the cell surface was measured by the addition of 1×10^5 HaCaT cells to a suspension, followed by the addition of either HPV16 or HPV18 (MOI = 25). Attachment to the ECM was measured by removing cells from slides with EDTA, leaving only the ECM. Either HPV16 or HPV18 (MOI = 25) was then added and allowed to bind for 2 h, followed by washes to remove any unbound particles. After 2 h, bound virus was quantified by qPCR against a standard curve of known concentrations of HPV genomes. (B) HaCaT cells were seeded and allowed to grow for ~2 days. Cells were then left untreated or treated with 10 mM EDTA to remove the cells. Cells were then stained with an antibody against laminin 5 (red) to identify the ECM and with Hoechst dye (blue) to identify the nucleus. (C) Either HPV16 or HPV18 was added to HaCaT cells for 2 h to allow binding, followed by washing of unbound particles. Cells were then stained with anti-L1 antibodies specific for HPV16 L1 (H16.V5) or HPV18 L1 (H18.J4) (green) and either CD147 or LN-5 (red). Nuclei were identified via Hoechst staining (blue). The inset in the merged image is representative of a magnified portion of the merged image (indicated by a small white box within the image). (D) The localization of attachment was quantified by using NIS-Elements Analysis software to analyze the

(Continued on next page)

examine this, we carried out attachment assays on cells, followed by coimmunofluorescence staining of bound virus particles and either the cell surface (CD147 stain) or the ECM (laminin 5 [LN-5]/LN-332 stain) (Fig. 6C). CD147 and LN-5/LN-332 were previously utilized to identify the cell surface and ECM (55, 84, 85). In agreement with data from the qPCR-based binding assay, HPV18 showed a preference for binding to the ECM compared to the cell surface (Fig. 6D). In contrast to the qPCR data, HPV16 showed a preference for binding to the cell surface as opposed to the ECM, with 82.1% of virions binding to the cell surface and 14.7% binding to the ECM (Fig. 6C and D). This discrepancy is likely due to both the cell surface and the ECM being present for the immunofluorescence assay as opposed to only one or the other in the qPCR assay.

We then wanted to determine whether the differential patterns of binding of HPV16 and HPV18 to the cell surface versus the ECM correlated with a difference in the rate of cell entry. HPV16 or HPV18 was synchronously bound to cells for 2 h at 4°C. Unbound virions were then washed away, and the cells were moved to 37°C to allow entry. At different time points postattachment, neutralizing antibodies against HPV16 and HPV18 were added to cells (Fig. 6E) (85). Virions bound to the cell surface are neutralized, while virions that have already been internalized will lead to infection. Neutralization is defined as a decrease in infection by at least 50% compared to a control infection. Infections in the absence of antibody were used as positive controls for infection, and antibody added at the time of infection ($t = 0$) served as a control for neutralization. At all time points postinfection, HPV18 was efficiently neutralized, indicating that much of the virus was still attached to the ECM or the cell surface. In contrast, HPV16 was efficiently neutralized only up to 12 h, indicating that at some time point between 12 and 24 h, the majority of virions were internalized, leading to infection. Taken together, these data suggest a correlation between the ability to directly bind to the cell surface and the rate of entry.

HPV16 L2 is involved in superinfection exclusion between HPV16 and HPV18. HPV16 and HPV18 differ in their abilities to bind to the cell surface in the presence of HSPGs, which is mediated mainly by L1 (66, 73, 75, 80, 82, 83, 86–90). Additionally, HPV16 and HPV18 are differentially dependent on cellular furin, which cleaves the N terminus of L2, allowing for conformational changes to the capsid that are required for infectious entry (74, 87, 91, 92). Chimeric viruses are an important tool for examining the role of specific viral proteins in the infectivity pathway and the role of specific proteins during competition between two virus types. To determine whether HPV16 L2 was responsible for the ability of HPV16 to block HPV18 attachment to the cell surface, chimeric viruses were generated by replacing HPV18 L2 with either full-length HPV16 L2 [HPV18-L2(16)L1(Δ18)] or portions of HPV16 L2 [HPV18-L2(18/16)L1(Δ18)] (Fig. 7). The chimeric viruses were previously generated and characterized by our laboratory (93, 94). The virions were generated from a HPV18 construct in which HPV18 L1 is translated from the consensus methionine residue (H18ΔL1) and thus is missing the upstream 61 amino acids. This L1 deletion has been shown to have no effect on HPV18 infectivity compared to that of wild-type (WT) HPV18 (data not shown). In a coinfection with WT HPV16 and the chimeric virus HPV18-L2(16)L1(Δ18), chimeric HPV18 was able to infect cells just as efficiently in the presence of WT HPV16 as in a single infection (Fig. 7A). In a coinfection with WT HPV16 and the chimeric virus HPV18-L2(18/16)L1(Δ18), where only the N-terminal region of HPV18 L2 is replaced, there was a small, but not significant, decrease in chimeric HPV18 infectivity in the presence of WT HPV16

FIG 6 Legend (Continued)
signal overlap between stained virions and a localization marker (CD147 or LN-5). (E) Either HPV16 or HPV18 (MOI = 10) was added to cells for 2 h at 4°C. Unbound virus was then washed, and neutralizing antibody was added at the indicated time points postinfection. Samples were harvested after 48 h, and qRT-PCR to analyze the E1^E4 splice transcript was done to measure infectivity. Panels A and E are representative of data from three individual experiments utilizing at least two different HPV16 and HPV18 preparations. Bars represent the standard deviations. An asterisk denotes significance by Student's *t* test. ns, nonsignificant. Statistical significance was defined as a *P* value of ≤0.05. Panel B is representative of data from two individual experiments. Panels C and D are representative of data from two individual experiments with at least two different HPV16 and HPV18 preparations. Images and quantifications are representative of results for at least 15 fields of view for each individual experiment, and standard deviations are shown for quantifications.

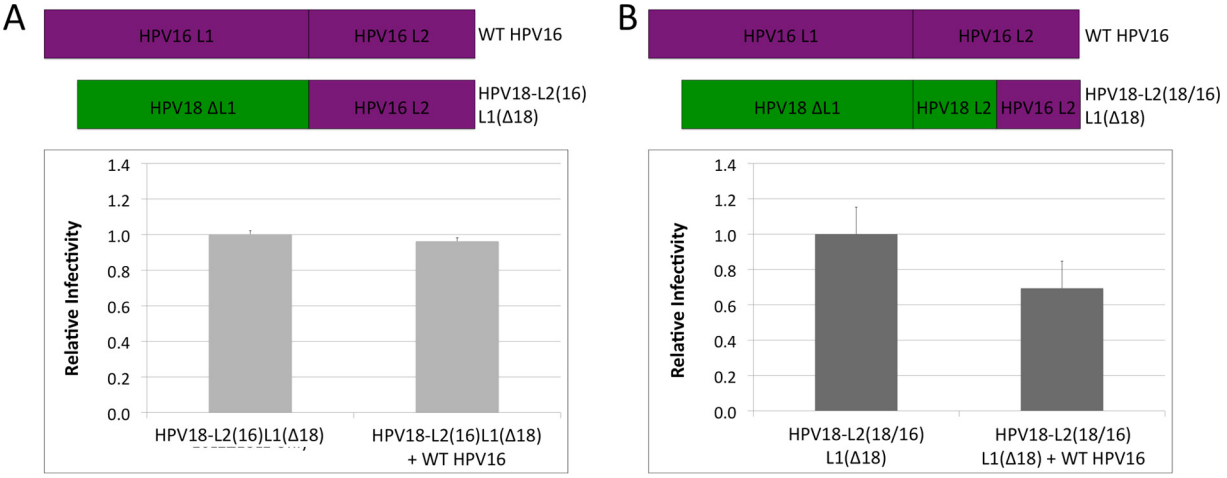


FIG 7 HPV16 L2 is involved in superinfection exclusion of HPV18 during a coinfection. HaCaT cells were infected with the chimeric virus HPV18-L2(16)L1(Δ18) only (A), the chimeric virus HPV18-L2(18/16)L1(Δ18) only (B), or both chimeric HPV18 and chimeric HPV16. At 48 hpi, mRNA was harvested, and infectivity was determined via qRT-PCR amplification of the E1∧E4 splice transcript. The levels of infection are relative, with values for single infections being set equal to 1. Data are representative of results from at least three individual experiments utilizing at least two different HPV16 and HPV18 preparations. Bars represent standard deviations. Statistical significance was defined as a *P* value of ≤0.05.

(Fig. 7B). These data indicate that HPV16 L2 is an important molecule in blocking HPV18 infectivity during a coinfection.

DISCUSSION

The phenomenon of SIE, in which one virus actively prevents a cell from being infected with another, usually related, virus is common. At least one mechanism of SIE has been demonstrated for many different virus types, with many viruses utilizing multiple mechanisms (1–15). Recent epidemiological studies of HPV infections have concluded that many women who are HPV positive are actually infected with more than one type of HPV (37–47, 95–97). Two previous studies of coinfections have been done, with conflicting results. The first study utilized the xenograft system and identified regionally separate areas of infection during a coinfection, indicating that single cells were unable to be infected with more than one HPV type (76). However, the detection of HPV is limited by the sensitivity of the technique utilized, and it cannot be ruled out that infected cells did not contain more than one type. In contrast, another study, in which cells were coelectroporated with linearized HPV genomes, identified cells that could efficiently replicate and maintain more than one HPV type in a single cell (98). While those studies examined the feasibility of coinfections, no mechanistic studies of competition or SIE between different HPV types have been done.

When HPV16 and HPV18 were simultaneously added to cells, single cells harboring both virus types were visualized. By using RNA-FISH to detect either E1∧E4 or E1-E2 mRNA, we were able to quantify the number of cells that were singly infected or coinfected with each HPV type. Using the Poisson distribution, the expected frequency of individual infections with either HPV16 or HPV18 was 99%. In cells that were coinfected, assuming that these are independent events, the expected frequency of cells being infected with both HPV16 and HPV18 was 98%. However, these predictions assume that each virus particle added to cells is equally infectious. As we did not attain this level of infection in individual infections or coinfections, it can be assumed that not all virions are infectious. While PsV has been reported to have a low infectivity/particle ratio (99), an infectivity/particle ratio for native HPV has not yet been determined. This experiment also confirmed that two HPV types could be simultaneously transcriptionally active within the same cell. When we measured the infectivity of HPV16 and HPV18 by qRT-PCR amplification of the E1∧E4 splice transcript, we saw a significant decrease in HPV18 infectivity in a coinfection compared to a single infection. This decrease in HPV18 infectivity was not observed when we analyzed coinfections with VLPs or QVs,

which may be due to a difference in the binding affinities between NV and recombinant particles or a difference in the particle/infectivity ratio. In the RNA-FISH assay, any cell that had at least one mRNA for either E1^E4 or E1-E2 was considered to be infected with that HPV type. However, the number of individual molecules of E1^E4 or E1-E2 was not quantitated. In contrast, the qRT-PCR assay measured total E1^E4 transcripts within a population of infected cells. Therefore, there are at least two possibilities that explain the decrease in HPV18 infectivity during a coinfection. The first possibility is that multiple HPV18 virions can enter the same cell and be transcriptionally active. Therefore, while the total numbers of infected cells are the same for HPV16 and HPV18, not as many HPV18 particles enter each cell due to HPV16 partially blocking or delaying the entry of HPV18. This would potentially lead to a decrease in the number of E1^E4 transcripts being produced and therefore would explain the results that we obtained by qRT-PCR. The second possibility is that HPV16 and HPV18 equally enter cells in the presence of each other but that HPV16 is able to partially block or interfere with HPV18 transcription, leading to a significant decrease in the number of HPV18 transcripts produced in cells that also contain HPV16. These results indicate that there is some degree of competition or SIE during a coinfection with HPV16 and HPV18.

In an attempt to restore HPV18 infectivity in the presence of HPV16, we infected cells with increasing amounts of HPV18. HPV16 was still efficiently able to block HPV18 infectivity when up to 5 times more HPV18 was added to cells, with the level of infectivity never returning to levels with a single HPV18 infection. The level of HPV16 infectivity remained constant, regardless of how much HPV18 was present. Likewise, we were unable to restore HPV18 infectivity by prebinding HPV18 to cells 1.5 h prior to the addition of HPV16. HPV can remain on the cell surface for up to 24 h postinfection (56, 100, 101). Furthermore, HPV16 and HPV18 have been shown to have different requirements for entry into cells (73–75, 102). Therefore, if HPV18 has not yet entered cells by the time that HPV16 is added, it is possible that HPV16 is able to block HPV18 on the ECM/cell surface, limiting HPV18 infectivity.

HPV18 infectivity was also blocked in cells acutely infected with HPV16 followed by superinfection with HPV18 after 24 h. In contrast, when HPV18 was added to cells first, followed by the addition of HPV16 24 h later, there was no decrease in HPV18 infectivity. In cells that harbor persistent infections, there is no block in the ability of HPV16 or HPV18 to superinfect cells. These data serve to further substantiate the idea that the mechanism of SIE is on the ECM/cell surface, when both virus types are present.

To rule out a block at the level of transcription, HaCaT cells were singly infected or cotransfected with HPV16 and HPV18. Transfection of the genomes directly into cells allows bypassing of receptor binding, internalization, and trafficking, allowing the virus equal access to the cell nucleus. Cells that were cotransfected had a decrease in the level of E1^E4 for both HPV types, compared to cells that were singly transfected. However, the E1^E4 levels of both types returned to the levels of a single transfection by 72 h posttransfection. This early decrease for both types is likely due to an initial competition for cellular resources and cellular transcriptional machinery. These data indicate that when both types are allowed equal, simultaneous access to the nucleus, they are equally transcriptionally active, and no SIE is present.

To directly assess a block in attachment, we carried out studies utilizing qPCR to identify particles bound to cells during a coinfection. While the quantity of HPV16 particles remained consistent, regardless of the presence of HPV18, the quantity of HPV18 particles bound was drastically decreased in the presence of HPV16. This was confirmed visually by immunofluorescence staining of the virions on the cell surface. It was previously demonstrated that HPV16 and HPV18 have different requirements for attachment and entry, with HPV16 infecting cells independent of cellular furin (74) and heparan sulfate proteoglycans (73, 75, 84). Due to these differences, it is possible that the initial interaction with the ECM or the cell surface varies among different HPV types (88). Additionally, it is possible that these results would vary depending on the cell type being utilized in the experiment. Importantly, a specific entry receptor for HPV has yet to be defined. For related polyomaviruses, it has been demonstrated that different

polyomavirus types utilize different cellular entry receptors (60, 103, 104). The utilization of different receptors for attachment and entry may allow SIE, whereby one virus type blocks another from gaining access to cellular receptors. We further show that HPV16 binds directly to the cell surface, bypassing the need for attachment to the ECM. In contrast, HPV18 preferentially binds to the ECM prior to infection. It is possible that since HPV16 is precleaved with furin and infects cells independent of HSPGs, HPV16 is able to bind directly to the cell surface and be internalized, whereas HPV18 needs to bind to the ECM prior to conformational changes and transfer to the cell surface, where L2 is then cleaved by furin. We show that HPV16 may be able to enter cells at a higher rate than HPV18, possibly due to its ability to bind directly to the cell surface.

One of the major differences in HPV16 and HPV18 is the necessity for furin during infection. The consensus HPV entry pathway indicates that furin cleaves the minor capsid protein L2, after attachment and before internalization, allowing for further conformational changes in the capsid structure and interactions with additional receptors (87, 100, 105). To assess whether HPV16 is able to block HPV18 infectivity due to its ability to infect cells in the absence of furin, we made chimeric viruses by swapping HPV16 L2 into the HPV18 genome. When coinfections with WT HPV16 and chimeric HPV18, which contains HPV16 L2, were done, HPV16 no longer blocked HPV18 infectivity. These data indicate that the L2 protein plays a role in the ability of HPV16 to block HPV18. However, the exact mechanism is unclear. A multitude of cellular receptors have been identified for HPV, including HSPGs (57, 58, 66, 88, 101), integrin $\alpha 6/\beta 4$ (61–65), growth factor receptors such as epidermal growth factor receptor (EGFR) and keratinocyte growth factor receptor (KGFR) (66), annexin-A2 (106), and the tetraspanin CD151 (107, 108). Of these receptors, attachment to $\alpha 6/\beta 4$ and EGFR/KGFR has been shown to induce signaling cascades, which could lead to changes in the cell surface to prevent the attachment of additional HPV virions. Also, the attachment of one HPV type could induce the downregulation of the receptor, thus preventing infection of the same cell with another HPV type.

In conclusion, we have identified a novel interaction between HPV16 and HPV18 whereby coinfection with both types leads to superinfection exclusion. While the exact molecular mechanism is unknown, we have shown that HPV L2 is at least partly responsible for this exclusion/competition.

MATERIALS AND METHODS

Ethics statement. The use of human cervical and foreskin keratinocyte tissue samples to develop cell lines as well as for infectivity assays for these studies was approved by the Institutional Review Board at the Pennsylvania State University College of Medicine. Discarded, deidentified tissue samples were exempt from the need for informed patient consent. Informed consent was waived by the Institutional Review Board.

Cell culture. HaCaT cells (kindly provided by Norbert Fusenig, DKFZ, Heidelberg, Germany) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 0.025 mg/ml gentamicin, and 0.11 mg/ml sodium pyruvate. Primary human keratinocytes from cervical biopsy specimens or foreskin circumcision specimens were isolated as described previously (109). Primary keratinocytes were maintained in 154 medium supplemented with a human keratinocyte growth supplementation kit (Cascade Biologics, Inc., Portland, OR). Immortalized keratinocytes, HPV16 WT:3 (110) and HPV18c (111), stably maintaining an HPV genome were maintained in E-medium with J2 3T3 feeder cells (112) and grown as previously described (112).

Production of native virus. Rafts were harvested and virus was isolated as previously described (113). Briefly, tissue was removed from the collagen plug and homogenized in phosphate buffer (0.05 M Na-phosphate, pH 8.0). Nonencapsidated genomes were removed by treatment with Benzonase (375 U; Sigma) at 37°C for 1 h. NaCl was added to a final concentration of 1 M, and the samples were centrifuged for 10 min at 10,500 rpm in a microcentrifuge to remove cellular debris. Supernatants were stored at −80°C.

Production of recombinant particles. HPV16 and HPV18 VLPs were generated in 293TT cells as previously described (114, 116). Briefly, 293TT cells were grown to 90% confluence in T-150 flasks, followed by transfection with HPV capsid protein plasmids only (VLPs) or with the capsid protein plasmid and WT HPV16 and HPV18 genomes (QVs) by using Lipofectamine 2000. The HPV16 plasmids were p16L1h (L1 expression plasmid) and p16L22h (L2 expression plasmid). The HPV18 plasmid was p18shell. Cells were split 1:2 at 24 h posttransfection and harvested at 48 h posttransfection. Cell pellets were resuspended in a total volume of 750 μl of phosphate-buffered saline (PBS). Cells were lysed by Dounce homogenization as described above for the HPV isolation protocol. $MgCl_2$ was added to a final concentration of 2 mM, followed by incubation of the lysates at 37°C overnight to allow maturation. In

the QV samples, unpackaged DNA was digested by the addition of 0.2% Benzonase (Sigma) and incubated for 1 h at 37°C. After digestion, NaCl was added to a final concentration of 1 M. The virus preparation was then centrifuged for 10 min at 10,500 rpm to remove cellular debris. The virus-containing supernatant was then collected and stored at −80°C.

Purification of virus particles by an OptiPrep gradient. Red purification of both NVs and recombinant particles by OptiPrep purification was preformed as described previously (114). Briefly, OptiPrep gradients were produced by underlaying (adding the most-dense OptiPrep solution to the tube first, followed by adding the less-dense OptiPrep solutions underneath) 27%, 33%, and 39% OptiPrep. Gradients were allowed to diffuse for 1 h at room temperature (RT). Next, 300 to 500 μl of clarified, Benzonase-treated virus preparations was layered on top of the gradient. Tubes were then centrifuged in an SW55 rotor (Beckman) at 234,000 $\times$ *g* at 16°C for 3.5 h. After centrifugation, 11 500-μl fractions were collected from the top of each tube. The titer of each fraction from NV and QV samples was determined by qRT-PCR as described below. For VLPs, Western blotting of each fraction was performed to determine the fraction(s) with the highest concentration of capsids.

Titration of virus. Viral genomes from NVs or QVs, either obtained directly from the virus lysate or purified by ultracentrifugation, were released from 10 μl of the virus preparation by resuspension in a solution containing 178 μl Hirt DNA extraction buffer (400 mM NaCl–10 mM Tris-HCl–10 mM EDTA [pH 8.0]), 2 μl of 20 mg/ml proteinase K, and 10 μl 10% SDS for 2 h at 37°C. The DNA was purified via phenol-chloroform extraction followed by ethanol precipitation and resuspension of the DNA in 20 μl Tris-EDTA (TE) (pH 7.4) (113). Titers were determined by utilizing a qPCR-based DNA encapsidation assay and a Qiagen QuantiTect SYBR green PCR kit. Viral genome amplification was carried out as previously described, using E2 primers with a standard curve of 10-fold serial dilutions (10^8 to 10^4 genome copies/μl) (113, 115). Titers of OptiPrep-purified VLPs were determined by a Bradford assay using the Pierce Coomassie (Bradford) protein assay kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Briefly, bovine serum albumin (BSA) protein standards with concentrations ranging from 0 to 2,000 μg/ml, the equivalent of a MOI of 10 of OptiPrep-purified NVs, and a series of dilutions of OptiPrep-purified VLPs were added to wells of a 96-well plate. Coomassie reagent was added to each well, and the plate was incubated on a plate shaker at room temperature. After 10 min, a plate reader was used to measure the absorbance of each well at 595 nm. The amount of VLPs equivalent to the amount of NVs at a MOI of 10 was then calculated.

Infections. HaCaT cells (50,000 cells/well) or healthy human primary keratinocytes (80,000 cells/well) were seeded into 24-well plates 2 days prior to infection. Virus was combined with medium in a total volume of 500 μl and added to cells. All infections were done at a MOI of 10, unless otherwise noted. Virus was incubated with the cells at 37°C with 5% CO_2 for approximately 48 h, followed by harvesting of RNA using a Qiagen RNeasy kit. Infections were analyzed by utilizing a qRT-PCR-based infectivity assay to measure the level of the HPV E1$\wedge$E4 splice transcript, as previously described (113, 115). Results are representative of means and standard deviations for at least three independent infections using at least two different batches of virus preparations for each virus type. Student's *t* test was performed, with statistical significance calculated as a *P* value of $\leq$0.05.

Attachment assays. Cells were seeded as described above for the infectivity assays. Cells were then incubated with virus (MOI = 25) in a total volume of 200 μl for 2 h at 4°C. Cells were washed twice with PBS to remove unbound particles, and DNA was harvested by incubation in Hirt lysis buffer for 2 h at 37°C, followed by phenol-chloroform extraction and ethanol precipitation. DNA pellets were resuspended in 20 μl TE (pH 7.4). The number of particles attached to the cells was determined by qPCR using primers targeted to E2 and measured against a standard curve, as described above for the encapsidation assay. Results are representative of data from at least three independent attachment assays of at least two different batches of virus preparations for each type. Student's *t* test was performed, with statistical significance calculated as a *P* value of $\leq$0.05.

To determine virus attachment to the cell surface only, cells were infected in suspension. HaCaT cells (150,000 cells/tube) were resuspended in 100 μl HaCaT medium and incubated for 2 h at 37°C with either HPV16 or HPV18 (MOI of 25 for both). Cells were then pelleted and washed twice with PBS to remove any unbound viral particles. Cells were then lysed, DNA was harvested, and attachment was analyzed as described above. Results are representative of data from at least three independent attachment assays of at least two different batches of virus preparations for each type. Student's *t* test was performed, with statistical significance calculated as a *P* value of $\leq$0.05.

To determine virus attachment to the ECM only, cells were seeded as described above for the infectivity assay. HaCaT cells were then removed by a 10-min treatment with 10 mM EDTA followed by pipetting, leaving only the exposed ECM on the plate (55). The ECM was then incubated with either HPV16 or HPV18 (equivalent of a MOI of 25) for 2 h at 37°C. The ECM was then washed to remove unbound virions, and DNA was harvested and attachment was analyzed as described above. Results are representative of data from at least three independent attachment assays of at least two different batches of virus preparations for each type. Student's *t* test was performed, with statistical significance calculated as a *P* value of $\leq$0.05.

Transfections. HaCaT cells were seeded as described above for the infectivity assay. Two days later, cells were transfected with HPV16, HPV18, or both HPV16 and HPV18 episomal DNAs by using Lipofectamine 2000 according to the protocol provided by the manufacturer. At 48 h posttransfection, mRNA was harvested and measured as described above for the infectivity assay. Results are representative of data from three independent transfections. Student's *t* test was performed, with statistical significance calculated as a *P* value of $\leq$ 0.05.

Immunofluorescence. HaCaT cells were seeded at 150,000 cells/well 1 day prior to attachment. Virus (MOI = 50) was then added to cells and allowed to attach as described above. After incubation, cells were washed three times with PBS to remove unbound particles. Cells were then fixed in 1% paraformaldehyde (PFA) for 5 min and blocked with 1% BSA–5% FBS–PBS-Triton X-100 for 1 h at RT. Virions were detected by staining with anti-L1 antibodies H16.V5 (1:1,000) and H18.J4 (1:1,000) diluted in blocking buffer for 1 h at RT. The ECM was detected via an anti-laminin 5 (laminin 332) antibody (1:1,000), and the cell surface was detected via an anti-CD147 antibody (1:50). Both H16.V5 and H18.J4 were kindly provided by Neil Christensen (Penn State College of Medicine, Hershey, PA). Cells were washed three times for 2 min each in PBS, followed by incubation with Alexa Fluor-labeled secondary antibodies (Invitrogen) for 1 h at RT. Cells were then washed once for 10 min in PBS with Hoechst dye, followed by three 2-min washes in PBS. Mounting medium (Invitrogen) and a coverslip were applied, and slides were allowed to dry overnight. Images were acquired with a Nikon Cf2 confocal microscope and a Nikon C2 camera. Quantitation of virion attachment localization was completed by utilizing Huygens Professional 15.10.0p8 software. Images are representative of results from two individual staining experiments utilizing two different virus preparations. Quantitation is shown as the mean from two individual staining experiments.

RNA fluorescent *in situ* hybridization. HaCaT cells were seeded and infected as described above for the infectivity assays or left uninfected. HPV16 and HPV18 cell lines were seeded at 60,000 cells/well 2 days prior to FISH staining. RNA-FISH was performed by utilizing the ViewRNA ISH cell assay kit from Affymetrix. RNA-FISH staining was done according to the manufacturer's instructions. Briefly, cells were washed in PBS and fixed in 4% PFA. Cells were then permeabilized in detergent for 5 min, followed by digestion with protease for 10 min at RT. Hybridization with the HPV16 and/or HPV18 E1^E4 or E1-E2 probe sets was then done for 3 h at 40°C. Following washes, hybridization with a preamplifier, followed by hybridization with an amplifier, and hybridization with label probe mixes were each performed for 30 min at 40°C. Cells were then washed and incubated with Hoechst dye for 10 min for nuclear staining, followed by additional washes and the addition of mounting medium and coverslips. Images were acquired with a Nikon Cf2 confocal microscope and a Nikon C2 camera. Quantitation of FISH staining was performed by utilizing Nikon Elements AR 4.30.01 software. Results are representative of data from two individual staining experiments utilizing two different virus preparations.

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